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THE PREPARATION OF STEM SECTIONS OF WOODY HERBARIUM SPECIMENS

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ABSTRACT.—**A procedure is described for sectioning the stems of woody herbarium specimens by the paraffin method. The specimens are cut into convenient sizes; boiled for one-half to one hour in order to exclude air and soak up the cell walls and membranes; cooled and placed in 5% NaOH for 24 hours in order to expand the collapsed cells and remove excessive coloring matter; washed in running water for a few hours; placed in hydrofluoric acid until sufficiently softened to cut easily with a razor blade; washed in running water for 24 hours to remove the acid; dehydrated and embedded in paraffin of high melting point (56°-58° C.) according to the n-butyl alcohol method; sectioned with the rotary microtome and completed by the ordinary method. Soaking the paraffin blocks in water for a period of several hours to a day or more before sectioning greatly improves the cutting and reduces electrification of the paraffin ribbon. The method proves satisfactory not only for herbarium material but for seeds and specimens of old bark and wood varying in hardness from balsa to ebony. For seeds or specimens containing only xylem, the NaOH treatment should be omitted.**

In determining the nodal condition of plants and in dealing with various other problems connected with stem anatomy, it frequently becomes necessary to resort to herbarium specimens for materials. The drastic treatment which the specimen undergoes during pressing and drying often results in the collapse of cells and crushing of the softer outer portions of the stem. By soaking or boiling the stems in water one may be able to get satisfactory sections of the woody portions, but the softer parts usually fall away or become badly torn during the sectioning process. Embedding in celloidin is the pro-

[1]The work reported in this paper was done at the Biological Laboratories, Harvard University, Cambridge, Mass.

cedure usually followed when complete sections are desired. Since this method is long and rather costly the writer has attempted to apply the *n*-butyl alcohol technic[2], making such adjustments as appeared necessary.

The first problem is to cut the dried herbarium specimen into convenient sizes. This may usually be done best with a sharp knife or razor blade while the specimen is dry, but if the bark is loose and flaky it may cut better after soaking a while in water. Care should be taken at this stage that the cells are not crushed or they will fall away and be lost.

Another problem is the restoration of the cells of specimens to an approximation of their normal shape and size. Since the flattened soft portions of the stem of pressed herbarium specimens are usually without excessive tissue distortion, it would seem that this restoration might be accomplished by means of a proper reagent. Considerable success is attained by treatment with NaOH and KOH. The range of concentrations most desirable is between 1 and 10%. Not only are the collapsed cells expanded but also much of the dark coloring matter commonly occurring in herbarium specimens is dissolved out. Staining is usually greatly improved by the clearing action of these reagents.

The next problem is the softening of the material prior to infiltrating with paraffin. A certain amount of softening action results from treatment with the above reagents but it is insufficient to soften hard materials. If the material contains an excessive amount of crystals or hard fibrous material a treatment with HF is necessary. The length of time required for the softening process will, of course, depend upon the nature of the specimen and the strength of the acid used. When the material cuts readily with a razor blade it should be removed from the acid. It is well to put an extra specimen in the acid to use as a test piece in order to avoid crushing the specimens intended for study.

The procedure from this point is that of the *n*-butyl alcohol method. (The celloidin technic may be employed here for those who favor this method and desire to use the sliding microtome.)

Another point which has to do with sectioning of especially difficult material is worth mentioning. It is well known that the cutting of paraffin-embedded material is often greatly improved by simply soaking the paraffin blocks in water for several hours or even for much longer periods. Certain materials, however, if left in water for

[2]Zirkle, Conway. 1930. The use of *n*-butyl alcohol in dehydrating woody tissue for paraffin embedding. Science, n. s., **71**, 103–4.

more than a day will swell and crack badly. Material which cuts poorly may cut well after this treatment. Furthermore, it is often found that this procedure greatly reduces the electrification of the paraffin ribbon. If the water treatment fails to soften the specimens, the following method may often be resorted to with success: simply trim the paraffin blocks down until the specimen is exposed and place the specimen (exposed side down) in a waxed jar of strong HF (40–52%) for a day or more; remove from the acid and wash in running water for a period of at least several hours before cutting. Thin sections of old corn stems, hardwood twigs and seeds may be cut by this method. The chief advantage of this technic is that it will apply to material which is already embedded in paraffin and which might otherwise be discarded as being impossible to cut. In general, however, it is better to soften the material sufficiently before embedding in paraffin, because otherwise sections of specimens with heterogeneous structure or with gelatinous fibers may crack badly upon being affixed to the slides and allowed to dry previous to the removal of paraffin.

Following is a tentative schedule for preparing sections of herbarium material:

1. Cut the specimens into pieces of convenient sizes, selecting only the portions needed for study. If the outer portions of the stem break or crumple, try cutting after the next step.

2. Boil for one-half to one and a half hours in order to exclude air and soak up the cell walls and membranes. If the material is especially soft and fragile a treatment with cold water may suffice.

3. Cool and place in 5% NaOH for 24 hours in order to expand the collapsed cells and remove excessive coloring matter.

4. Wash in running water for a few hours. (Prolonged washing at this point is probably unnecessary because no insoluble salts should be formed with the acid of step 5.)

5. Soften in strong (40–52%) HF until the specimen can be cut easily and smoothly with a razor blade.

6. Wash in running water for 24 hours to remove the acid.

7. Dehydrate and embed in paraffin of high melting point (preferably 56°–58° C.) according to the *n*-butyl alcohol method.

8. Trim the paraffin blocks and soak in water for at least a few hours before sectioning with the rotary microtome.

9. If the cutting is still difficult place the paraffin blocks in HF for a day or more. Remove and wash well in running water overnight or longer before sectioning. (Step 9 should not be necessary and in fact may not prove satisfactory in all cases.)

10. Proceed from this point according to the ordinary method.

The above procedure has been tried with considerable success on soft, light woods (*Ochroma*) and such hard materials as: *Nothofagus*, *Quercus*, *Grubbia*, *Octoknema*, and *Cneorum* twigs; *Fraxinus*, old wood and bark; and dried heartwood of *Diospyros ebenum*.

For specimens containing only xylem, steps 1–4 may be omitted. Simply boil the blocks and proceed with step 5.

It appears that it should be possible to cut any plant material in paraffin by the above method.

STAINING PARAFFIN SECTIONS WITH PROTARGOL

6. IMPREGNATION AND DIFFERENTIATION OF NERVE FIBERS IN ADRENAL GLANDS OF MAMMALS

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Abstract.—**A series of experiments with protargol staining of nerve fibers in mammalian adrenal glands has yielded the following procedure: Fix 1-2 days in a mixture of formamide (Eastman Kodak Company) 10 cc., chloral hydrate 5 g., and 50% ethyl alcohol 90 cc. Wash, dehydrate and embed in paraffin. Cut sections about 15μ and mount on slides. Remove the paraffin and run down to distilled water. Mordant 1-2 days in a 1% aqueous solution of thallous (or lead) nitrate at 56-60°C. Wash thru several changes of distilled water and impregnate in 1% aqueous protargol (Winthrop Chemical Company) at 37-40°C. for 1 to 2 days. Rinse quickly in distilled water and differentiate 7-15 seconds in a 0.1% aqueous solution of oxalic acid. Rinse thru several changes of distilled water for a total time of 0.5 to 1.0 min. Reduce 3-5 min. in Bodian's reducer: hydroquinone 1 g., sodium sulfite 5 g., distilled water 100 cc. Wash in running water 3-5 min. and tone 5-10 min. in a 0.2% gold chloride solution. Wash 0.5 min. or more and reduce in a 2% oxalic acid solution to which has been added strong formalin, 1 cc. per 100. (Caution. This last reduction is critical and over-reduction can spoil an otherwise good stain; 15-30 seconds usually suffices, and the sections should show only the beginning of darkening to a purplish or gray color.) Wash, fix in hypo, wash, dehydrate and cover.**

During a study of the innervation of the adrenal gland, we have had an opportunity to experiment with its staining by Bodian's copper-protargol method (1937), by the 2-hour method (Davenport, McArthur and Bruesch, 1939) and by a number of modifications of impregnation, differentiation and reduction based on these methods. Differentiation of protargol stains by treatment with dilute acid before reduction of the silver was mentioned by Young (1939). In such differentiation, protargol acts like a basic dye, and in our hands proved to be a very effective means of destaining the glandular background. Since the step seemed to be important, we tried several organic acids in varying concentrations to determine the optimum type of treatment. Other experiments were made on the effect of adding copper to the protargol staining solution after the tissue had

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[2]Contribution No. 329.

been in it for 1 day. This "delayed copper" procedure combined with acid differentiation has given better stains than those obtained by earlier methods. Mordanting sections with nitrates before impregnating with protargol was tried also to observe differences between the effect of silver nitrate and nitrates of other heavy metals.

Experimental

1. *Source of tissue and method of fixation.* Adrenal glands from the following mammals were used: monkey, dog, cat, rat, guinea pig, mouse, cow and man. All fixation was for 1–2 days in formamide (Eastman Kodak Co.) 10 cc., chloral hydrate 5g., and 50% ethyl alcohol 90 cc. This fixative was decided upon as a result of the experiments made on fixation by Bank and Davenport (1940). It has given consistent results in the staining of nerve fibers and yielded tissue blocks which cut easily. Embedding in paraffin, cutting (15μ sections) and mounting were done in the customary manner.

2. *Impregnation.* The following variations were tried:

a. Protargol 0.5 and 1% solution for 24 and 48 hr. at 37° C.

b. Protargol as in *a*, 24 hr., then 10 cm. of 20-gauge copper wire wound or folded to fit the bottom of a Coplin jar dropped in and the impregnation continued for another 24 hr.

c. Treatment with 1% solutions of nitrates of the following metals for 24 and 48 hr. at 60° C.: cadmium, copper, lead, silver, thallium (monovalent), and uranium. This "mordanting" was followed by washing and then impregnating 48 hr. at 37° C. in protargol solution.

d. Protargol plus copper for 48 hr. at 37° C. as in Bodian's method.

e. Silver nitrate solution for 24 hr.; then copper-protargol for 24 hr.

3. *Acid differentiation.* Oxalic acid was used in concentrations of 0.05, 0.1, 0.2, 0.5 and 1% aqueous solution and the slides treated for various periods of time from 5 seconds to 1 minute. Fumaric, formic, acetic, lactic, benzoic and hydrochloric acids were used in 0.1% concentration for 5 to 15-second periods of treatment.

4. *Reduction.* Amidol, bromhydroquinone, and hydroquinone as 1% solutions in 5% sodium sulfite were tried and, in the case of amidol and hydroquinone, a few sections were reduced with solutions to which 2 cc. and 4 cc. of glacial acetic acid were added to each 100 cc.

5. *Subsequent processing.* In the usual manner sections were washed thoroly after reduction, toned in 0.2% gold chloride, washed again and treated with 2% oxalic acid in 1% aqueous formalin solution. A final fixing in hypo, washing, dehydrating and covering in balsam completed the process. It was found that the reduction in formalin-oxalic solution was highly critical and care had to be taken that this step was not overdone (usually not over 15 to 30 seconds)

Results

Impregnations as listed under 2*a*, *b* and *d* above were successful. The value of the various metallic nitrates used as "mordants" (2*c*) came out in the following order: thallium (monovalent), lead, cadmium, copper, uranium, and silver. Thallium and lead nitrates improved the impregnation and differentiation of nerve fibers. Cadmium nitrate did no harm but was little or no better than a distilled water control or plain protargol without pretreatment. The last three, copper, uranium, and silver nitrates, gave poor stains of nerve fibers. Silver nitrate followed by copper-protargol (2*e*) failed. In comparing *a*, *b* and *d* it was evident that copper acted as a differentiating agent by suppressing the staining of connective tissue and enhancing cell nuclei and nerve fibers. The effect of copper was not the same as the acid differentiation and its use tended to nullify the subsequent effect of the acid. Acid differentiation without the use of copper destained cell nuclei. Addition of copper after the impregnation in plain protargol had been in progress for a day was an effective means of modifying its action, but sections so treated required acid differentiation (fig. 3).

The optimum concentration of oxalic acid used for differentiating after impregnation was found to be 0.05 to 0.1%. The weaker organic acids worked more slowly in 0.1% concentration but gave final results similar to oxalic acid. Hydrochloric acid did not differentiate, presumably because silver chloride is very insoluble. The time needed for actual immersion of the slides in 0.1% oxalic acid varied from 5 to 20 seconds. The optimum had to be determined by trial for the various tissue blocks, but was usually about 10 seconds. Washing for 0.5 to 1.0 minute thereafter thru two or three changes of distilled water helped to prevent surface precipitates. The time of washing was not critical but apparently should not be over 1 minute.

Amidol-sulfite solutions were quite unsuited for reduction because they gave a general stain without much differentiation of nerve fibers. Bromohydroquinone gave results similar to hydroquinone altho it was somewhat more vigorous in action and the final stains tended to be darker. Hydroquinone as used in Bodian's method was the most satisfactory reducer. The addition of acid to the reducing solution was not advantageous in amidol solutions and it caused complete failure with hydroquinone. Therefore, acidification of the reducing solution was not a substitute for the acid treatment before reduction.

Discussion and Summary

On the basis of the foregoing experiments we are more than ever impressed by the uncertainty of applying to one type of tissue a

Plate 1. Photomicrographs which illustrate the staining of nerve fibers in the adrenal gland.

Figs. 1, 2 and 3. Sympathetic ganglia of the adrenal capsule of dog, stained as follows: 1. protargol, hydroquinone; 2. protargol, oxalic acid differentiation, hydroquinone; 3. protargol, copper-protargol, fumaric acid, hydroquinone. All ×500. Figs. 2 and 3 show the improvement effected by acid differentiation.

Fig. 4. Nerve fascicle at the cortico-medullary junction, dog; silver nitrate, protargol, oxalic acid, hydroquinone. The very pale background and reduction of fiber staining intensity resulted from the silver nitrate treatment.

Fig. 5. A similarly located fascicle in monkey; protargol, oxalic acid, hydroquinone.

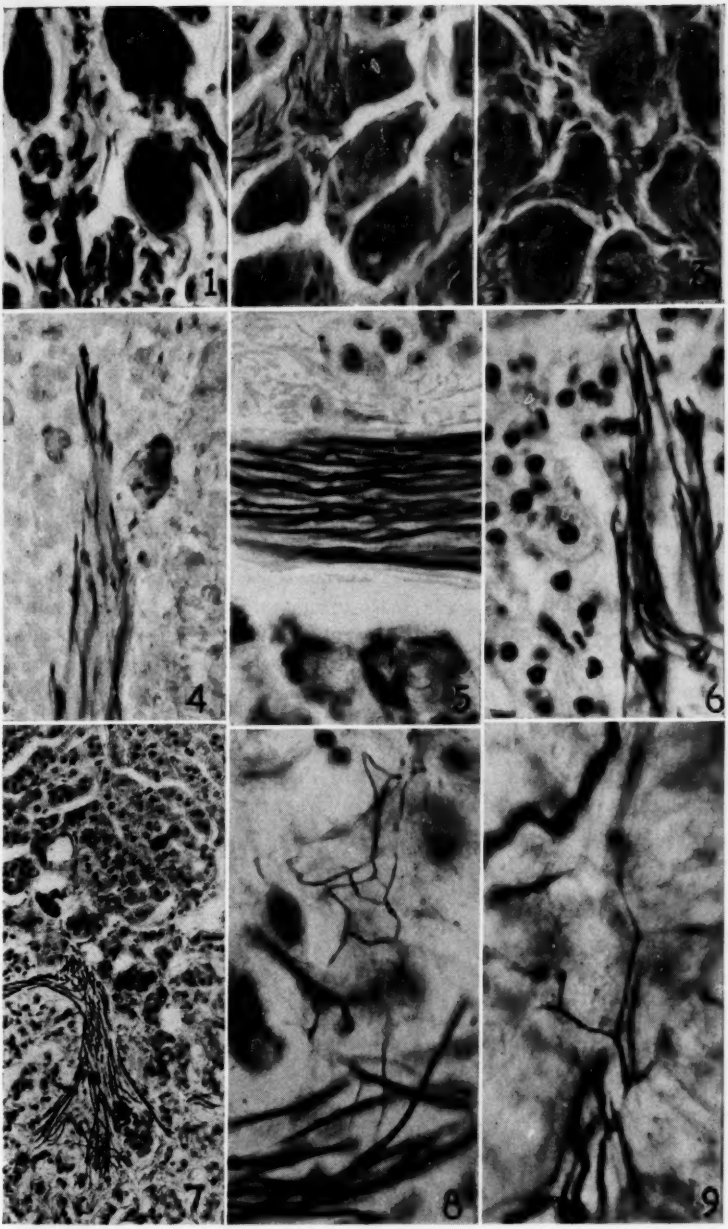
Fig. 6. Intracortical fibers in dog; protargol, fumaric acid, hydroquinone.

Figs. 4, 5 and 6, ×500.

Fig. 7. Fascicle at the cortico-medullary junction in cat; thallium nitrate, protargol, oxalic acid, hydroquinone. ×145.

Fig. 8. The same slide as Fig. 7 except photographed at a magnification of 1000 to show the just resolvable pericellular fibers in the medulla.

Fig. 9. Similar to Fig. 8, excepting that the pretreatment was with lead nitrate instead of thallium and the specimen is the adrenal medulla of dog. ×1000.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silver staining technic which has been worked out for a different type. The arbitrary procedure given in the abstract of this paper may succeed with glandular tissue other than the adrenal gland, but we have not tried it with other glands. The sympathetic ganglia adjacent to the gland stained well when the medullary fibers stained well (Figs. 2 to 5), hence it should be suited to sympathetic nerves elsewhere.

Acid differentiation in a dilute (0.1%) aqueous solution of an organic acid between the steps of protargol impregnation and reduction contributed much toward successful staining of fine nerve fibers in the adrenal medulla. Without the acid treatment, the tissue reduced to a dense brown, but with increasing degrees of acid differentiation background colors ranging progressively thru reddish brown, pink, lavender and blue were obtained. The color served as a guide to the time required in the acid bath. Reddish pink medullary tissue with lavender cortex and black nerve fibers was optimum.

The question of the value of mordants preceding protargol impregnation is not settled but rather merely raised by these experiments. In view of the results obtained with lead or thallium nitrate (Figs. 7 to 9), they seemed to have improved the impregnation of fine nerve fibers and warrant the recommendation of their use. There may be other salts which would influence the selectivity of protargol.

When fixation was made with formamide-chloral-hydrate solution, the use of copper with the protargol bath was optional, and when used, was preferably added later. On the basis of some earlier experiments it appeared that the addition of copper was essential to nerve fiber impregnation after fixation in formalin.

The modifications which differed from earlier protargol methods consisted of mordanting with lead or thallous nitrate solution in a paraffin oven preceding protargol impregnation, and the use of a carefully controlled acid differentiating bath after the impregnation.

Note: Sections should be affixed to the slides as flat as possible and be dried thoroly to minimize loosening during the rather long staining process.

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BANK, E. W., and DAVENPORT, H. A. 1940. Staining paraffin sections with protargol. 5. Chloral hydrate mixtures, with and without formamide, for fixing peripheral nerves. Stain Techn., **15**, 9–14.

AN IMPROVED TRIMMER FOR PARAFFIN BLOCKS

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In January 1937, the author published in *Stain Technology* a description of a simple trimmer for paraffin blocks. Since that time so many requests have been received for dimensions and diagrams that it was decided to build a new instrument incorporating several additional features and eliminating certain characteristics which had proved to be undesirable.

The object of the instrument (Fig. 1) is to trim the paraffin block so that the opposite edges will be strictly parallel, thereby preventing the ribbon from curling as it comes off the microtome. This is of particular advantage when using small blocks or when serial sections are desired. While the original design was satisfactory in this respect, there were some undesirable defects. The most outstanding of these was the absence of any device to compensate for side motion when the sliding parts should become worn.

The new instrument has a T-shaped bed made of cast iron. The moving parts are of bronze and are mounted on the upper surface of the bed within steel key-ways, and fastened by machine screws. One side of each key-way is fastened solidly to the bed by flat-head bright, machine screws. The opposite side is attached by cylindrical head screws which pass thru oversize holes permitting compensatory adjustments when side motion, occasioned by use, appears. The blades are made from suitable lengths of stainless steel strips which are ground and honed to a razor edge. These blades are mounted upon the blade carriers by means of set screws. The holes in the blades are also oversize to permit adjustments. The blade carriers are moved into position by means of finely threaded screws which have large knurled heads; the carriers are tapped and threaded to receive these screws.

The microtome pedestal is mounted in a sleeve which permits the paraffin block to be rotated thru 90 degrees. This insures squaring of the block so that the sections will spread evenly and without cracking when placed upon a warm stage or floated upon warm water. The sleeve in turn is mounted in a carrier which is attached to a brass plate by a single large shoulder screw. This permits the carrier to be oriented with reference to the blades. The whole assembly runs in the keyway perpendicular to the blades.

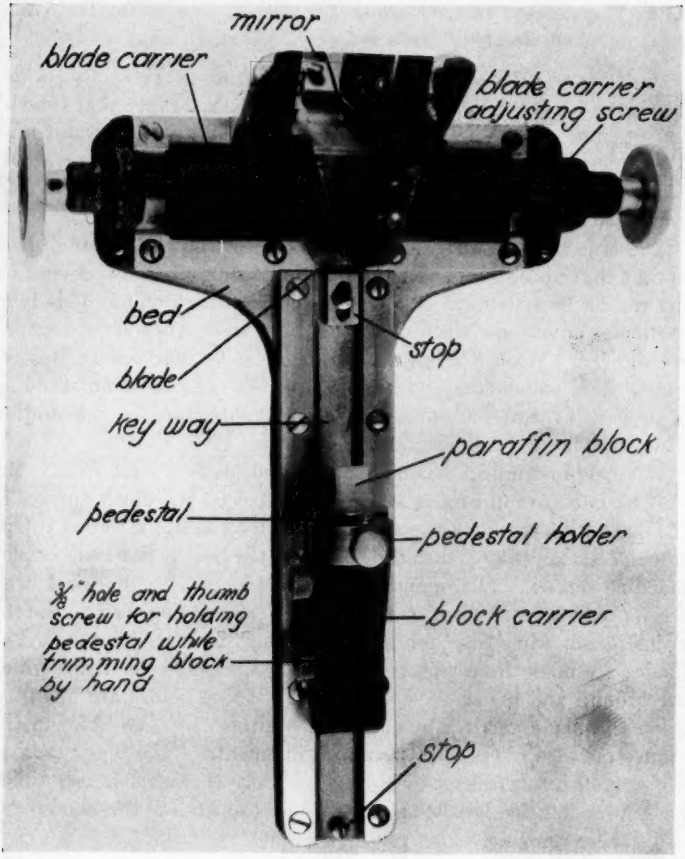

Fig. 1. Photograph of instrument with a paraffin block in position for trimming.

It is advisable to trim the block by hand before using the trimmer. To facilitate this a hole with an accompanying thumb screw is drilled vertically on the back of the carrier. The pedestal can thus be held

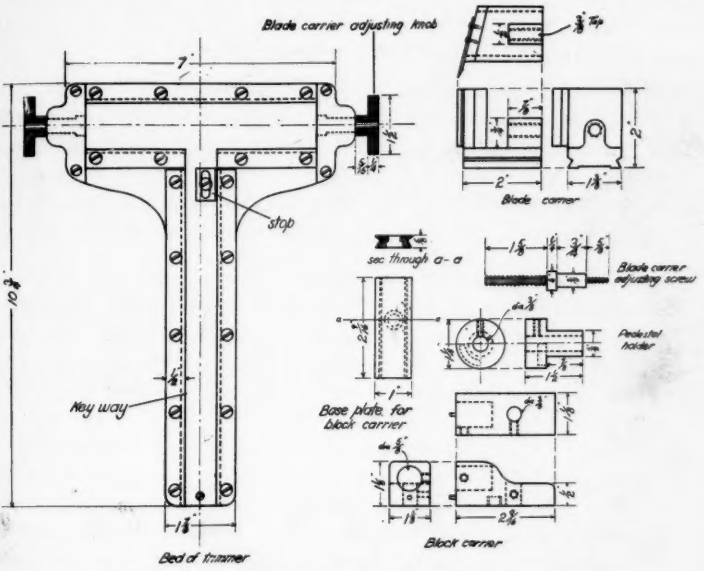

Fig. 2. Working drawing showing all important dimensions.

solidly and in a vertical position while the block is being trimmed by hand.

A stainless steel mirror is mounted on the front of the instrument. This permits the operator to observe the position of the tissue in the block.

The author wishes to express his appreciation to Mr. Carl Spriegle, instrument maker of the Georgetown University Department of Physics, for aid in construction.

STAINING SCAB ACTINOMYCES IN POTATO TUBER TISSUES

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ABSTRACT.—**Actinomyces hyphae imbedded in the middle lamellae of potato tuber cells may be stained in sections by the use of a modified Gram's stain. The modifications are: a very strong (5%) solution of crystal violet in anilin oil; a 24-hour exposure to both the dye and the iodine solution; and a slow decolorization in absolute alcohol until no more color flows.**

Potato scab *Actinomyces* filaments may be readily traced in pieces of the thin skin stripped from small tubers, mounted whole in 10% glycerin without cover glass, allowed to dry for several days with 10% glycerin, with 50% glycerin finally added. The mycelium secretes its typical dark-brown pigment into the adjacent cell walls and can be followed.

The location of the filaments in tuber sections presents such difficulties that no complete account has yet been given of the extent to which they penetrate the tubers, nor their location in the cells.

Potato-infesting *Actinomyces* species are Gram-positive in the living portions of the mycelium; the dead regions are Gram-negative.

Stains, such as Flemming's triple, are not differential, in that they stain nucleus, cytoplasm, and cell walls, as well as any enclosed parasites. The desired stain will affect the parasite alone so that the plant tissues are left unstained. Gram's stain offered possibilities since *Actinomyces* filaments from cultures retain the stain even after a long wash in 95% alcohol. Tests made on microtome sections of young potato tubers following the ordinary Gram method resulted in complete decolorization both of potato tissue and of any parasitic filaments, if the stain solution was that ordinarily used and if the time was the same or longer.

A trial was then made of Jones' method for Gram-positive bacteria.[1] This method is really the triple stain plus the application of Gram's iodine solution and a very strong gentian violet solution. Results with us were still unfavorable, the parasite seemed to decolorize as readily as the host tissues. The use of the strong gentian violet solution of Jones' stain was retained but with the following procedure.

[1]Jones, F. R. Winter injury of alfalfa. J. Agr. Research, 37, 189–211. 1928.

Small potatoes preserved in 50% alcohol (or Allen-Bouin's mixture) were imbedded in paraffin and sectioned about 8–10 μ thick. The mounted paraffin sections were handled as follows:

1. Treatment with xylol, absolute alcohol, 95% alcohol, and then washing in water.

2. Staining in the gentian violet (crystal violet) solution for 24 hours. This solution was made as follows: 10 cc. of 95% alcohol and 2 cc. of anilin oil were shaken thoroly and then 88 cc. of distilled water added. In this mixture, 5 g. of crystal violet (as small crystals as possible) were dissolved. The solution was shaken thoroly, allowed to stand for a day and then filtered.

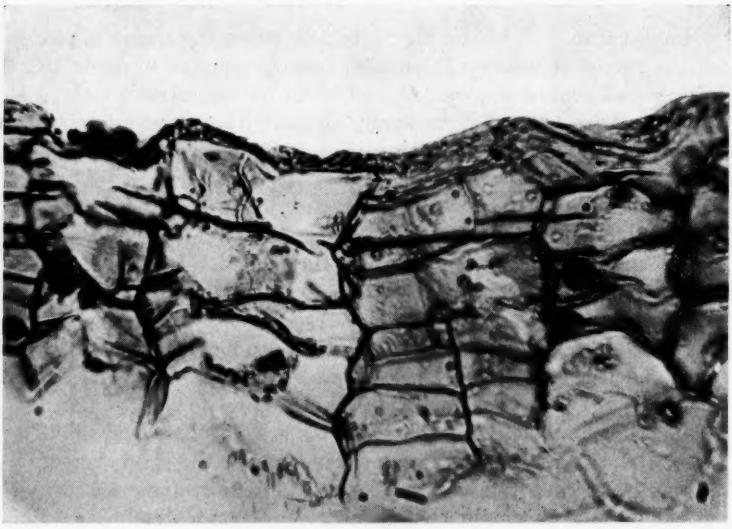

Fig. 1. Section of young potato tuber cork layer; Actinomyces filaments stained bl e, host walls, colorless. ×500.

3. Washing in water to remove excess stain.

4. Immersion in Gram's iodine solution (iodine 1 g., KI 2 g., distilled water 300 cc.) for 24 hours.

5. Washing in absolute alcohol until no more color flowed out of the sections.

6. Immersion in xylol until no further color was drawn from the sections. If none appeared, the sections were mounted in Canada balsam dissolved in xylol.

This preparation of the solutions follows Rawlins' directions[2] rather than those of Jones, who simply states, "the sections were stained according to Gram's method as described in manuals of bacteriology except that each slide was dipped in water after immersion in the iodine solution to prevent the appearance of iodine crystals after dehydration."

Weigert's Gram stain,[3] used for differentiation of Gram-positive bacteria in animal tissues, one part of anilin oil to two parts of xylol, was tried; but the action was so rapid that all gentian violet was removed. It could not be poured on and off the sections without complete destaining. Decolorization in absolute alcohol required up to an hour which gave an opportunity to control its action.

The results were satisfactory in the large majority of trials. Two difficulties were encountered: (1) A complete bleaching of the sections, altho the cells were infected; cause still undiscovered. (2) Gentian violet continued to run in small amounts into the balsam, altho decolorization had been apparently complete in absolute alcohol, making the balsam surrounding the sections a faint purple. The organisms could, however, be seen clearly differentiated in the tuber cell walls.

In the best preparations the organisms were a deep, bluish-black with the host cell walls unstained. The walls of the cork layer had a faint yellow tinge and could be readily seen without a counter-stain, so none was used.

The hyphae of the organism push their way thru the middle lamellae of the potato cells, as has been noted by other investigators and as can be readily seen in views of surface mounts. In stained sections, this relation of potato cell walls to the hyphae is not so clear; stained portions seem to be cell walls but are really the enclosed hyphae; while unstained portions occur where the hyphae have twisted out of focus or the branches of the hyphae inside the walls have been cut off in sectioning.

The hyphae vary considerably in diameter and some are so fine as to be almost on the line of visibility with the oil immersion lens. These very fine filaments decolorize if the destaining is carried too far while the larger ones retain their color. The composition of the enclosing potato cell walls has also an effect on the rapidity of the destaining process. Cork walls are relatively impermeable and the

[2]Rawlins, T. E. Phytopathological and Botanical Research Methods. Wiley, New York. 1933.

[3]Weigert, C. Über eine neue Methode zur Färbung von Fibrin und von Microorganismen. Fortzchr. d. Med., 5, 228–32. 1887.

rather large hyphae enclosed in them make destaining rather slow. The cellulose walls of the body of the tuber do not protect the delicate hyphae enclosed and destaining is more rapid. The consequence is that a single preparation is very likely to be better either for the large hyphae imbedded in the cork cell walls or for these deeper in the tuber, altho preparations may be obtained where all *Actinomyces* hyphae retain a brilliant color.

EFFECT ON TISSUE VOLUME OF VARIOUS METHODS OF FIXATION, DEHYDRATION, AND EMBEDDING.[1]

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ABSTRACT.—**A new indirect method is described for following volume changes of homogeneous pieces of tissue during fixation, dehydration and embedding, and area changes during sectioning, staining and mounting. Pieces of rabbit kidney cortex were compared after fixation in Destin's, Orth's, Petrunkevitch's cupric-paranitrophenol, Bouin's, SUSA, Zenker-formol, 10% formalin in distilled water, formalin in saline, Burke's pyridine formalin, $CaCO_3$-neutralized formalin, $MgCO_3$-neutralized formalin, Bensley's vacuum distilled neutral formalin in distilled water, and Bensley's neutral formalin in saline; during dehydration in ethyl alcohol, dioxan, and tertiary butyl alcohol and clearing in xylol and chloroform; and after infiltration and embedding with parowax, with paraffin-nitrocellulose double embedding technic, with hot nitrocellulose, and with cold nitrocellulose. The H-ion concentration of these fixatives was followed during tissue fixation.**

Altho all fixatives showed specific differences, SUSA and Bouin's gave the best general results and neutral formalin mixtures, especially pyridine-formalin, the poorest. Isotonic saline was found superior to distilled water as a formalin diluent, reducing tissue swelling during formalin fixation. Reagents producing marked decreases in tissue volume render such tissues less susceptible to shrinkage during subsequent procedures. Shrinkage of tissue during dehydration and infiltration with hot paraffin may exceed that produced by fixation alone. Excessive heat causes tissue distortion and shrinkage. Infiltration with hot paraffin causes considerable shrinkage, with hot nitrocellulose less, and with cold nitrocellulose the least shrinkage.

In the preparation of microscopical sections living tissues undergo certain changes. These vary with different methods employed. The following experiments were undertaken to compare some of the main factors concerned in these changes.

[1]Appreciation is expressed for the suggestions of Professors H. Kirkman and J. E. Markee of the Department of Anatomy and Mr. J. G. Beckerley of the Department of Physics at Stanford University.

[2]At present at the Barnard Free Skin and Cancer Hospital, St. Louis, Missouri.

Methods of Volume Determination

For the studies on changes in tissue volume, the cortex of rabbit kidney from which the capsule had been stripped was cut into pieces about 0.5 × 0.5 × 0.3 cm. By using small pieces of this tissue, complete, uniform, and rapid penetration of fluids was obtained. The change in volume of the tissue was determined indirectly by observing the change in area of one surface of the block. With an unaltered optical system (Fig. 1) consisting of a low power compound microscope, giving an initial magnification of ten diameters, and a camera lucida, successive tracings were made of the same surface of the tissue block

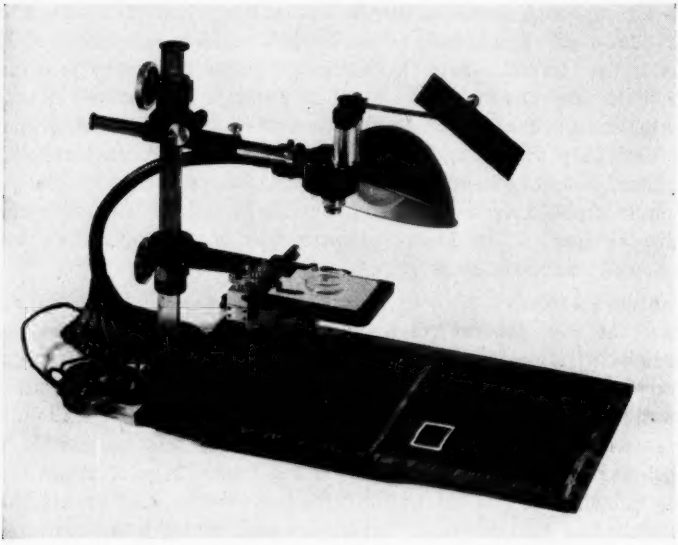

Fig. 1. Apparatus for indirect volumetric comparisons.

barely covered by different fluids. To minimize and standardize the spherical aberration, the block of tissue was centered and oriented in the optical field in the same position each time with the assistance of an eyepiece micrometer and a mechanical stage which held a glass plate upon which rested the dish of fluid with the immersed tissue. To improve the definition of the camera lucida image, the drawings were made with white pencil upon black paper. The area of the tracings was found with the aid of a planimeter, and a volume factor for determining the volume change for any given piece of tissue was obtained by taking the area reading of the planimeter to the 3/2

power. It is desirable to convert the area readings to a volume factor because the changes in area are not the same as the changes in the volume.

Since for valid results, changes in the tissue block dimensions must be uniform, care was taken to use only blocks which did not change in shape. The constancy of the optical system was checked frequently by making tracings of a standard object. Changes in the thickness of the tissue blocks resulted in changes in magnification of the optical system due to the variation in distance from the objective lens to the tissue block. Consequently, at the conclusion of the experiment the thickness of the firm tissue blocks was determined with fine calipers, and previous area readings corrected accordingly. The thickness of the tissue at previous area readings was found by using a nomograph solving the equation, $\log A_1/\log A_2 = \log T_1^2/\log T_2^2$, in which A refers to area and T to thickness of the tissue, with the subscript numbers designating the various area readings. T_1–T_2 gave the change in tissue thickness. The variation in magnification per millimeter change in thickness was found by experimentally altering the distance from the objective to the object. Usually three area tracings were made of each of two to five comparable tissue blocks at each stage of the experiment and the average of two or three planimeter determinations of each of the six to fifteen tracings taken as the area.

The indirect method of determining volume change is especially adaptable to use upon small pieces of homogeneous tissue for which accurate direct volume determining apparatus is not available. Tarkhan (1931) and others have mentioned the fact that if tissues are permitted to dry between fluid changes, they show a greater shrinkage. With this method the tissues are always covered by the proper fluid, and volume comparisons can even be made in melted paraffin, celloidin, balsam, or any other clear or semi-clear liquid or solid medium. By keeping the covering media at a constant, minimal thickness, it is not necessary to make a correction for differences in refractile properties of the media. The coefficient of variability was 2.80 for the volume changes of twelve pieces of tissue (Fig. 3; No. 1, 2, 6, and 7) during formalin fixation.

Comparing the newly presented indirect method of volume determination with the direct method using displacement by tissues of fluid in a burette, four pieces of rabbit kidney cortex were found to have volumes, as compared with their fresh volumes, of $140\pm3\%$ after fixation in 10% formalin, of $102\pm12\%$ in 70% alcohol, and of $60\pm6\%$ after embedding in parowax. Volume values were taken

TABLE 1. THE pH READINGS, TO THE NEAREST 0.05 pH, OF VARIOUS FIXING FLUIDS, EACH CONTAINING ONE-TWENTIETH OF ITS WEIGHT IN DOG KIDNEY

Fixing Fluid	Fresh sol. pH	pH-value at various periods after fixation started										
		Hours							Days			
		1	2½	5	8	18	26	49	6	17	95	175
10% formalin (Baker's) in distilled water	4.35	4.70	5.20	5.40	5.45	5.45	5.60	5.60	5.60	5.10	4.85	4.40
10% formalin in 0.9% sodium chloride	4.35	4.80	4.80	5.10	5.10	5.15	5.15	5.15	5.10	4.80	4.60	4.40
Burke's pyridine formalin (25%)	8.20	7.90	7.60	7.80	7.80	7.75	7.70	7.70	7.70	7.60	7.55	7.25
$CaCO_3$ 10% neutralized formalin	6.60	6.30	5.90	5.95	5.90	5.85	5.80	5.80	5.75	5.40	5.05	4.85
$MgCo_3$ 10% neutralized formalin	7.15	6.70	6.20	6.20	6.20	6.10	6.05	6.00	5.95	5.60	5.20	4.95
10% Bensley's neutral formalin	6.85	6.35	6.40	6.25	6.25	6.15	6.15	6.15	6.10	5.70	5.25	5.00
10% Bensley's neutral formalin in saline	6.90	6.25	5.80	5.80	5.80	5.75	5.75	5.60	5.40	5.10	4.85	4.70
10% formalin (Merck's) in distilled water	6.50	6.45	6.00	6.00	5.90	5.90	5.90	5.80	5.80	5.30	5.15	4.60
Destin's fluid	3.90	—	3.80	3.80	3.80	3.80	3.85	3.85	3.90	3.90	3.90	3.85
Orth's fluid	4.00	—	4.20	4.50	4.50	4.80	—	5.00	—	—	—	—
Zenker-formol	3.70	3.85	4.00	4.00	4.10	4.20	4.15	4.15	4.25	4.25	—	—
Bouin's fixative	2.00	—	2.00	2.00	2.00	2.00	2.05	2.00	2.05	2.05	2.05	2.05
SUSA fixative	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.15	1.20	1.20	—	—

from the average of two separate burette readings, which checked within 0.02 cc., the volume of the fresh block varying from 0.14 cc. to 0.30 cc. A repetition of the determination for the average volume of five pieces of fixed tissue checked within 1%.

The kidney cortex after removal from the rabbit was kept in fresh rabbit serum for the short time before the camera lucida tracings of the fresh tissue blocks could be completed. The experimental work on volume was conducted in two separate parts, the second being performed several months after the first, using tissues from a different animal and different reagents; hence the two parts are not directly comparable. All tissue samples for each part of the experiment were taken from the same animal to insure uniform consistency.

2. pH Changes of Fixing Fluids During Tissue Fixation

Several freshly excised pieces of dog kidney totaling about 2.5 cc. were weighed to within 0.02 g. and placed in twenty times their volume of fixative in chemically clean glass-stoppered vials. The pH-values of fixing fluids (Table 1) were taken with a glass electrode just before the tissues were dropped into them, at intervals during fixation, and, in some cases, for 6 months subsequently.

During formalin fixation the changes in pH were greatest within the first 5 hours. During the first 48 hours the pH-value of all solutions tended to approach a common median point, probably the reaction of the tissue, altho the solution containing pyridine had the greatest buffer capacity and was altered least. After several days, however, the pH-value of all solutions started to fall slightly, probably from the breakdown of the formalin to give acidic compounds such as formic acid.

Zenker-formol and Orth's fixatives showed a moderate increase in pH. SUSA, Bouin, and Destin[3] showed relatively little change in their low pH-values.

3. Volume Changes During Various Fixations and Dehydrations

In the first part of the experiment on changes in tissue volume, the results of which are shown in Table 3 and Fig. 2, several samples were fixed in each of the following fluids: Petrunkevitch's No. 2 cupric-paranitrophenol, Bouin's, SUSA, Destin's, Orth's, Zenker-formol, and 10% formalin, dehydrated thru the alcohols to xylol, and embedded in parowax. Formalin-fixed groups were also dehydrated and cleared by each of the tertiary butyl alcohol, chloroform, and

[3]Dustin (?). Formula used in this study was taken from McClung, "Microscopical Technique," second edition.

dioxan methods. To get maximal effects of the various agents, the tissues were left for what was considered to be an ample length of time in each solution.

The tissue blocks were treated separately according to the following schedules. Tissues, after 16 hours in Petrunkevitch's fixative, were washed in 70% alcohol. After 18 hours in Bouin's, the tissue was transferred directly to 70% alcohol and given several changes. After 6 hours in Heidenhain's "SUSA" fluid, the tissue was washed in 70% alcohol. Tissue was fixed 7 days in Destin's fluid, washed 2 hours in distilled water, and upgraded to 70% alcohol. Following 5 days fixation in Orth's fluid, the tissue was washed 24 hours in running water and dehydrated to 70% alcohol. Samples were fixed 6 hours in Zenker-formol, washed in running water 16 hours, and upgraded to 70% alcohol. Following 48 hours fixation in 10% Merck's "neutral" formalin in distilled water, the tissues were washed 3 hours in running water and dehydrated to 70% alcohol. All tissues were left 24 hours or longer in 70% alcohol before further dehydration.

TABLE 2.— TERTIARY BUTYL ALCOHOL DILUTION TABLE (AFTER JOHANSEN)

	Percentages of TBEA			
	70%	85%	95%	100%
Distilled water	30 cc.	15 cc.	——	——
95% ethyl alcohol	50 cc.	50 cc.	45 cc.	——
Tertiary butyl alcohol	20 cc.	35 cc.	55 cc.	75 cc.
Absolute ethyl alcohol	——	——	——	25 cc.

The samples which were to go thru xylol were left 10 hours in 85% alcohol, 12 hours in 95% alcohol, a total of 4 hours in three changes of absolute alcohol, 1 hour in equal parts of absolute alcohol and xylol, a total of 3 hours in three changes of xylol, and 12 hours in xylol saturated with paraffin at 38°C. Tissues infiltrated with paraffin by the chloroform method were treated in the same way except that chloroform was substituted for xylol in the above mixtures. Samples were transferred from 70% alcohol to three changes of dioxan totaling 10 hours and then into a mixture of equal parts of dioxan and parowax for 12 hours at 50°C.

The tertiary butyl alcohol (TBA) schedule (table 2) provided for 24 hours in 70% tertiary butyl and ethyl alcohol (TBEA) mixture following transfer from 70% ethyl alcohol, 2 hours in 85% TBEA mixture, 2 hours in 95% TBEA, 2 hours in 100% TBEA, a total of 4 hours in three changes of pure TBA, 1 hour in a mixture of equal parts of TBA and paraffin oil, and then infiltration with parowax. This was done in the method suggested by Johansen (1940) by immersing the tissues in sufficient TBA-paraffin oil mixture on top of melted parowax, three-fourths filling shell vials which had been cooled enough to solidify the parowax. The vials were placed in the oven at 50°C. for 12 hours, and the tissues slowly settled as the parowax melted, thereby giving a gradual infiltration with parowax. The tissues were subjected to slightly greater heat than usual so as to show the shrinking effect of the heated reagents. All tissues during infiltration with parowax were given four changes of pure parowax totaling 5 hours at 52°C. and then embedded.

The TBA had a flash point of 70°, melting point of 17.5°C, and was obtained from California Botanical Materials Co., 787 Melville Ave., Palo Alto, Calif. The dioxan was from General Biological Supply House, Chicago, Ill.

4. Volume Changes with Various Formalin Fixations and Embeddings

In the second part of the experiment on changes in tissue volume, the results of which are shown in Table 4 and Fig. 2, three samples were fixed in each of the seven following fluids: 10% formalin in distilled water, 10% formalin in 0.9% aqueous NaCl, as suggested by Mann (1902, p. 92) and Carleton (1922), Burke's (1933) pyridine neutralized 25% formalin, $CaCO_3$-neutralized 10% formalin, $MgCO_3$-neutralized 10% formalin, Bensley's (1938, p. 43) neutral 10% formalin, and Bensley's neutral 10% formalin in 0.9% sodium chloride. Samples of formalin fixed tissue were embedded by each of the parowax, double embedding, hot nitrocellulose, and cold nitrocellulose technics.

The nitrocellulose used was made by Hercules Powder Company, Parlin, New Jersey and was labeled R. S. ½ second, viscosity 3/20. Altho "neutral" reagent formaldehyde of Merck & Co., Rahway, New Jersey, was used in the first part of the experiment, in the second part the C.P. analyzed formaldehyde of J. T. Baker Chemical Co., Phillipsburgh, New Jersey, was used as a stock solution. The same schedule for formalin fixation, alcohol dehydration, xylol clearing, and parowax infiltration and embedding was followed in all experiments. In the double embedding schedule tissues were dehydrated into absolute alcohol by the standard method and left in equal parts absolute alcohol and ether for 18 hours. After being transferred to 5% nitrocellulose for 3 days, they were put into a mixture of equal parts of 10% nitrocellulose and pure clove oil for 24 hours. The tissues were then dropped into two changes of chloroform of one-half hour each, into benzol for one hour, and then into benzol saturated with parowax at 38°C. for 15 hours. Infiltration was completed by four changes of parowax totaling 5 hours at 52°C.

In the hot nitrocellulose technic tissues were run thru absolute alcohol and ether as for double embedding and then thru three grades of nitrocellulose at 56°C. according to the specifications of Koneff and Lyons (1937) except that the times in the solutions were doubled because of the size of the tissues and the desire to permit ample time for determining the effects of the hot nitrocellulose.

With the cold nitrocellulose schedule, the tissues were upgraded thru absolute alcohol and ether as for double embedding and then thru solutions of 5, 10, and 20% nitrocellulose with 3 days in each. Tissues were embedded in 20% nitrocellulose, exposed to chloroform vapor for 2 hours, immersed in chloroform for 4 hours, and stored in 80% alcohol.

Using a modification of the apparatus shown in Fig. 1, in which the objective stage was moved to maintain the object surface at a constant distance from the objective lens despite the varying depth of the object, the area of the cut surface of one nitrocellulose block (Fig. 2, No. 2) was compared with that of the last three 16μ sections cut from it. Under standard optical conditions, as compared with the area of the block surface covered with 80% alcohol and a cover slip, the cut sections showed an area of 92±0% in 80% alcohol, of 82±0%

TABLE 3.—AVERAGE VOLUME PERCENT, AS COMPARED WITH FRESH TISSUE, OF RABBIT KIDNEY DURING FIXATION, WASHING, DEHYDRATION AND CLEARING, AND PAROWAX INFILTRATION AND EMBEDDING

(The number of samples in each group is shown in parentheses. The variation is expressed as the difference between the average volume percent and the greatest deviation from the average)

Procedure (see p. 72)	Fixed tissue	Washed tissue	Tissue in 70% alc.	Cleared tissue	Embedded tissue
Petrunkevitch-xylol	117±2(3)	———	130±1(3)	79±1(3)	64±4(3)
Bouin-xylol	86±1(2)	———	82±0(2)	71±3(2)	63±2(2)
SUSA-xylol	82±2(4)	———	83±4(4)	70±3(4)	58±2(4)
Destin-xylol*	104±3(3)	102±2(3)	91±3(3)	71±2(3)	56±4(3)
Orth-xylol	71±3(3)	67±3(3)	69±2(3)	64±1(3)	55±3(3)
Zenker-formol-xylol	70±4(4)	68±4(4)	61±5(4)	54±4(4)	47±1(4)
10% formalin TBA	129±2(3)	125±3(2)	78±2(3)	68±2(3)	60±3(3)
10% formalin-chloroform	133±2(4)	———	85±4(4)	74±3(4)	58±5(4)
10% formalin-xylol†	134±4(4)	126±1(2)	82±4(5)	64±2(4)	56±3(5)
10% formalin-dioxan	132±5(3)	———	84±5(3)	74±5(3)	39±4(3)

*Destin-xylol: in xylol-parowax, 67±1(2); in parowax 1½ hr. at 52° C., 63±1(2); in parowax 5 hr. at 52° C., 60±1(2).

†10% formalin-xylol: in absolute alcohol, 74±4(5); in parowax 5 hr. at 52° C., 57±3(3).

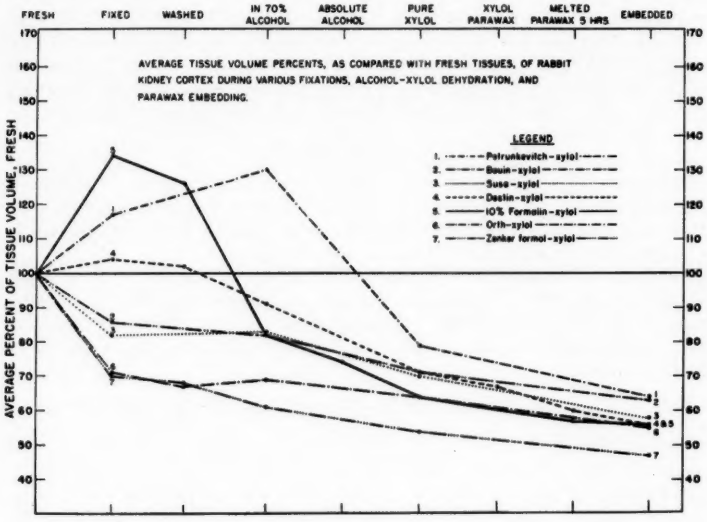

FIGURE 2

in xylol after staining the sections without fastening to slides, and of 82±0% after being mounted 16 hours in neutral balsam at 40°C.

A similar comparison between a block of formalin-fixed parowax-embedded kidney and five 8μ sections cut from it did not show any significant change in area. By the same method a decrease in area of 28±0% was found in comparing three 3μ mounted stained sections of Destin-fixed, TBA-dehydrated rat kidney with their block embedded in 62° paraffin. The tracings showed twice as much decrease in width as in length suggesting that compression during sectioning was an important factor, altho it was not evident macroscopically or microscopically.

Sections, 8μ thick, were cut at 25–28°C. from all groups of three comparable blocks in the first part of the experiment and in the second part from single, promptly fixed pieces submitted to each separate schedule, eliminating any difference in the microscopic picture due to varying delay before fixation. Altho several months elapsed between the time the tissues in the first part of the experiment were embedded and the time they were sectioned, all other blocks were sectioned a few days after embedding. Care was taken thruout the work to seal all blocks not being used by dipping in liquid parowax.

The relative ease with which about fifteen sections could be cut at a uniform speed from blocks previously trimmed and oriented for ready sectioning, was studied, especial attention being paid to the sound produced by the microtome knife passing thru the tissue. Since these small, well infiltrated tissues cut without difficulty, a somewhat dull knife was used in an attempt to detect maximal differences in sectioning and in tissue hardness. Since Aumonier (1938), in studying the distortion of tissues by sectioning, found the sharpness of the knife to be the chief variable, numerous blocks were sampled at the same place on the knife so that the sharpness of the knife would be relatively uniform.

To eliminate personal bias in judging ease of sectioning or the microscopic picture, blocks were assigned numbers and their identity obscured until the final results were tabulated. This comparison between blocks was repeated, varying the order of sectioning, so that each block was tested three times and rated according to ease of sectioning. Results for each method of treatment were averaged and arbitrarily divided into fairly distinct groups (Table 5).

Sections from each of the paraffin blocks were mounted and stained simultaneously in the same staining dish with Harris' hematoxylin and eosin and with Heidenhain's azocarmine modification of Mallory's triple connective tissue stain (Mallory, 1938). Sections were cut

TABLE 4.—AVERAGE VOLUME PERCENT, AS COMPARED WITH FRESH TISSUE, OF RABBIT RENAL CORTEX DURING FORMALIN FIXATION, ALCOHOL-XYLOL DEHYDRATION, AND EMBEDDING

(The number of samples in each group is shown in parentheses. The variation is expressed as the difference between the average volume percent and the greatest deviation from the average)

Procedure (see p. 73)	Fixed tissue	Tissue in 70% alc.	Absolute alcohol	Tissue in benzol	Embedded tissue
Formalin-cold-nitrocellulose	140±4(3)	108±2(3)	98±1(3)	———	92±2(3)
Formalin-hot-nitrocellulose	140±2(3)	105±2(3)	94±1(3)	———	79±4(3)
Bensley's neutral formalin-paroWax	166±7(3)	119±5(3)	———	———	74±2(2)
$CaCO_3$ neutralized formalin-paroWax	148±2(3)	108±2(3)	———	———	72±2(3)
$MgCO_3$ neutralized formalin-paroWax	146±5(3)	105±2(3)	———	———	68±3(3)
Formalin-paroWax	143±2(3)	109±3(3)	———	———	68±4(3)
Formalin double-embedding	141±5(3)	104±3(3)	97±2(3)	88±3(3)	68±4(3)
Bensley's neutral formalin in saline-paroWax	130±6(3)	93±4(3)	———	———	64±2(3)
Formalin in saline-paroWax	119±0(3)	87±4(3)	———	———	55±2(3)
Burke's pyridine-formalin-paroWax	170±0(2)	75±2(3)	———	———	48±2(3)

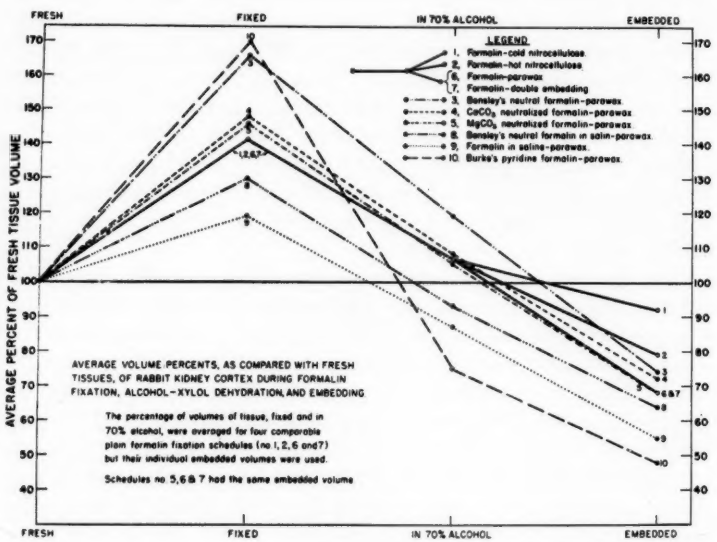
FRESH
FIXED
IN 70% ALCOHOL
EMBEDDED
AVERAGE PERCENT OF FRESH TISSUE VOLUME
LEGEND
1. Formalin-cold nitrocellulose.
2. Formalin-hot nitrocellulose.
6. Formalin-paroWax.
7. Formalin-double embedding.
3. Bensley's neutral formalin-paroWax.
4. $CaCO_3$ neutralized formalin-paroWax.
5. $MgCO_3$ neutralized formalin-paroWax.
8. Bensley's neutral formalin in salin-paroWax.
9. Formalin in saline-paroWax.
10. Burke's pyridine formalin-paroWax.
AVERAGE VOLUME PERCENTS, AS COMPARED WITH FRESH TISSUES, OF RABBIT KIDNEY CORTEX DURING FORMALIN FIXATION, ALCOHOL-XYLOL DEHYDRATION, AND EMBEDDING
The percentage of volumes of tissue, fixed and in 70% alcohol, were averaged for four comparable plain formalin fixation schedules (no 1, 2, 6 and 7) but their individual embedded volumes were used.
Schedules no 5, 6 & 7 had the same embedded volume

FIGURE 3

from the nitrocellulose blocks at 16, 12, and 8μ and stained similarly. The sections stained by each method, identified only by number, were carefully studied and graded (Table 5). In many cases tissue sub-

Table 5. — A comparison of the average hardness of tissue blocks and staining properties, fixation and differential shrinkage based (A) on three observations on each of three comparable mounted sections of rabbit kidney fixed and dehydrated by the various methods; and (B) on six observations on one mounted section each of rabbit kidney fixed in various formalin solutions and embedded by different methods.

For each method the hardness of tissue was averaged from three sectionings of each of three comparable blocks in (A) and for three sectionings of one block in (B). H&E is for Hematoxylin and Eosin and MAz for Mallory azan stain. Because of differences in technique sections stained and cut in nitrocellulose are not comparable with paraffin embedded material.

	PROCEDURE	MICROSCOPIC PICTURE									TISSUE HARDNESS
		NUCLEAR			CYTOPLASMIC				SHRINKAGE		
		Fixation	H&E Stain	MAz Stain	Fixation	H&E Stain	MAz cytoplasm	MAz c.t.	Glomerular	Tubular	
A	Petrunkevitch-xylol	++	+++	+++	+++	+++	+++	+++	Max.	Med.	Soft
	Bouin-xylol	+++	+++	+++	+	++	++	+++	Min.	Min.	Soft
	Susa-xylol	+++	+++	+++	++	++	+++	+++	Min.	Min.	Medium
	Destin-xylol	++	++	+++	+++	++	++	+++	Min.	Med.	Medium
	Orth-xylol	+	+	++	++	+	++	+++	Min.	Max.	Hard
	Zenker-formol-xylol	+++	+++	++	++	+	++	++	Min.	Max.	Soft
	10% Formalin-TBA	++	+++	+	+	+	+	+	Max.	Min.	Medium
	10% formalin-chloroform	++	+++	+	+	+	+	+	Max.	Min.	Medium
	10% formalin-xylol	++	+++	+	+	+	+	+	Max.	Min.	Medium
	10% formalin-dioxan	++	+++	+	+	+	+	+	Med.	Min.	Hard
B	Formalin-cold nitrocellulose	+++	+++	+	++	+++	++	+++	Min.	Min.	Hard
	Formalin-hot nitrocellulose	+++	+++	+	++	+++	++	++	Min.	Min.	Medium
	Bensley's formalin paraffin	+++	+++	+	+	+++	++	+	Med.	Med.	Medium
	$CaCO_3$ neutral. form. paraffin	+++	+++	+	+	+++	++	+	Max.	Med.	Hard
	$MgCO_3$ neutral. form. paraffin	+++	+++	+	+	+++	+++	++	Max.	Med.	Soft
	Formalin-paraffin	+++	+++	++	++	+++	+++	+++	Med.	Med.	Medium
	Formalin-double embedding	+++	+++	++	++	+++	+++	+++	Med.	Med.	Hard
	Bensley's form. in saline-para.	++	+++	++	++	+++	+++	++	Max.	Med.	Medium
	Formalin in saline-paraffin	+++	+++	++	++	+++	+++	+++	Med.	Med.	Hard
	Burke's pyridine form. paraffin	+	++	+	++	++	+++	++	Med.	Max.	Medium

jected to the same schedule held the same rank within each group thruout all six or nine total repeated testings. Results were checked by the independent rating of a competent cytologist.

Altho the tissue fixed in Petrunkevitch's fluid stained well, the nuclei appeared swollen and showed less distinct chromatin. This fixation gave the softest blocks for cutting. Bouin and SUSA gave the best all around results, altho their cytoplasmic fixation, especially Bouin's, was not so good. Their picture with Mallory-azan was the most brilliant. SUSA-fixed kidney showed the least differential shrinkage between glomerular loops and Bowman's capsule. By their distinctive glomerular picture in which the hyalin base of the

capillary loops stood out clearly and relatively distended, Destin-fixed sections were readily identified. Kidney fixed in Orth's fluid tended to stain diffusely with anilin blue. Tissues dehydrated with dioxan in all instances stained slightly poorer than formalin-fixed kidney dehydrated by other methods. Longer staining with Mallory-azan of formalin-fixed sections improved the connective tissue staining, but not the nuclear and cytoplasmic differentiation. Kidney fixed in neutralized formalin mixtures showed the poorest Mallory-azan staining in all respects and poorer cytoplasmic pictures than the more acid formalin solutions. The use of normal saline instead of distilled water as a formalin diluent improved cytoplasmic fixation, altho the tissue was somewhat harder for sectioning. Kidney fixed in Bensley's neutral formalin in distilled water showed an especially poor cytoplasmic fixation, but the glomerular picture with clear, open capillaries was definitely superior, resembling Destin-fixed glomeruli. The formalin-double-embedding schedule gave results equal or slightly superior to formalin-parowax in most respects, altho it gave slightly more glomerular shrinkage. Burke's pyridine formalin gave bizarre cytoplasmic swelling, which almost filled the proximal convoluted tubules.

5. General Discussion

FIXATION

The studies of the fixing agents reported in the literature show considerable variation in results. This is explained in part by the use of different tissues, of various sized blocks, of different schedules, and of reagents of not exactly the same composition.

Hirsch and Jacobs (1926) concluded that various chemical and physical properties of a fixative are more important than its molecular concentration. Altho the present results tend to confirm this finding, formalin solutions in isotonic saline were in general equal or superior to hypotonic formalin fixing solutions. Altho formalin solutions in saline gave less initial tissue swelling during fixation, the reaction of subsequent dehydration and embedding reagents produced shrinkage nearly equal to that of formalin without saline so that the total final shrinkage with saline was greater. This greater tissue shrinkage and density may explain in part the increased hardness on sectioning.

Burke (1931) has discussed the importance of H-ion concentration and the various methods of neutralizing formalin fixatives. Altho, as claimed by Burke, pyridine-formalin has better buffering and shows less change in pH during fixation, it gave outstandingly poor micro-

scopic results upon the kidney. Authorities differ as to the most desirable pH for formalin fixing solutions. Jacquiert (1930) working with snail hermaphrodite gland concluded that the best fixation is produced at pH-values much lower than those corresponding to the different isoelectric points of the various cellular elements. Manstein (1934) left mouse tissues, including kidney, in phosphate buffer solutions for 24–48 hours at various temperatures. The karyolytic and staining changes in the nuclei became much more pronounced as the reaction of the buffers was varied from pH 5 to 8. The present results show that, in general, ordinary acid formalin is superior to neutralized formalin for fixation of tissues such as kidney, altho on other tissues and for special purposes this might not be true. Most authors have their own preference as to the most desirable type of neutral formalin and it is very likely that each may have slight advantages. Generally, of the neutral mixtures tried, $MgCO_3$-neutralized formalin, followed closely by $CaCO_3$-neutralized formalin, gave the best results.

Sato (1931, I) reported that the higher the pH-value of the tissue the more the swelling. The present results for change in gross volume also show the same thing, with the exception of $CaCO_3$- and $MgCO_3$-neutralized formalin.

These studies show that for rabbit renal cortex "formalin shrinkage" is not caused by the fixative, as claimed by some, but by the dehydration and embedding. Several writers have stated that formalin does not seem to fix tissue proteins well, thus permitting alteration by subsequent reagents. For this reason, formalin-fixed tissues were chosen for a comparative dehydration and embedding study. Such tissues show extreme swelling and shrinking and give a poorer, more distorted microscopic picture than do tissues showing less extreme volume changes such as those fixed in SUSA, Bouin's, or Destin's fluids.

Since the renal cortex is made up of cellular and connective tissue elements which react differently to different reagents, as shown by Tarkhan and others, a reagent such as formalin would be expected to cause great differential shrinkage and distortion within the tissue. This was confirmed microscopically, since glomerular shrinkage was most marked with formalin fixed tissues. The fixative which produces the least volume change does not, however, necessarily give the best microscopical picture, for Zenker-formol, which caused the greatest shrinkage, is widely used because of its excellent cytoplasmic preservation. The most distinctive changes in the microscopic pic-

ture and gross volume were due to differences in fixation rather than dehydration. These experiments confirm the statements of others that tissues shrinking greatly during fixation tend to show less change under subsequent shrinking conditions.

DEHYDRATION AND CLEARING

Dioxan, which recently has been hailed by some as the answer to all tissue dehydration difficulties, caused 35% shrinkage during parowax infiltration, which is 3½ times the shrinkage during infiltration after xylol and 5 times the shrinkage during infiltration after TBA. It is possible that less shrinkage might have resulted if the tissue had not been dehydrated to 70% alcohol for purposes of comparison before going into dioxan or if the dioxan used had been dried over $CaCl_2$ before use, as recommended by some authorities, but not mentioned in some articles on dioxan. The dioxan used was supposedly free from water and satisfactory for tissue dehydration purposes. Johansen (1937) is one of the few writers to condemn, for other than toxicological reasons, the indiscriminate use of dioxan. He mentions that some brands contain at least 10% of water and other impurities which may produce poor paraffin infiltration and shrinkage.

Seki (1937, II) gave experimental evidence for the sluggish penetration and shrinkage effects of dioxan. Upon cow liver, spleen, and tendon, the measured gross shrinkage after dioxan was much greater than after ethyl alcohol. The results given here check with his observation that altho xylol shrinks tissues more than dioxan, in going into hot paraffin the final shrinkage is greater in dioxan treated tissue.

A block of formalin-fixed, dioxan-dehydrated kidney infiltrated in dioxan-parowax at 34°C. shrank less and was twice as soft for sectioning than were two comparable blocks left in the same mixture at 50°C., altho their microscopic pictures were similar.

The changes from 70% alcohol to xylol produced twice as much shrinkage as the transition from 70% alcohol to TBA, dioxan, or chloroform, all of which produced similar degrees of change. Chloroform permitted more change than xylol while the tissues were being infiltrated with parowax, but this may be explained partially by the greater initial shrinking action of xylol, thus permitting less shrinkage by the hot parowax subsequently. The least shrinkage in passing from 70% alcohol to final embedding and also in passing from complete dehydration to final embedding was obtained with TBA. After experience with all four methods of dehydration employed, it has been found that ethyl-alcohol-chloroform and especially TBA dehydration give the most satisfactory results in general micro-

technical work both in ease of sectioning and in final microscopic quality of tissues. Xylol has been the most unsatisfactory. Other things being equal, it is desirable to get minimal tissue shrinkage during fixation, dehydration and embedding.

EMBEDDING

A comparison of embedding methods shows comparable gross shrinkage with parowax and with parowax-nitrocellulose, less shrinkage with hot nitrocellulose, and slight shrinkage with cold nitrocellulose. Excessive heat, whether in nitrocellulose, dioxan-parowax, or especially in parowax, causes tissue shrinkage and should be avoided.

In cooling from 59° to 20°C., 500 cc. of parowax was found to decrease 14.3% in volume. The paraffin, in shrinking more than the tissue, must exert a compressing force upon it. Since during embedding celloidin itself may be observed to shrink slightly, one would expect the embedded tissue, as observed in the experiments with nitrocellulose, also to shrink slightly.

MICROSCOPIC SECTIONS

Embedded tissue has not necessarily reached its maximum capacity to shrink, so that in sectioning it may be further distorted, and subsequent heat, staining, dehydration, and clearing agents, which caused change in the volume of the tissue block, may result in still further change in the fragile section of tissue. Such changes occur during the sectioning and dehydration of nitrocellulose embedded sections; this has also been shown by Sugita (1917).

Aumonier (1938) found that thinner sections and "wax" of higher melting point gave greater shrinkage of tissues during cutting and fixing sections to the slide. This agrees with the present observation of shrinkage in thin sections embedded in hard paraffin while thicker sections in soft parowax, which were allowed only 30 seconds to straighten over water on a warming plate at 39°C., showed no area change. Sugita (1917, p. 523) also mentioned the importance of temperature while stretching paraffin ribbon and observed that, while at the melting point the section size was unaltered, excessive heat produced much expansion followed by sudden contraction to less than the original size on cooling. Since he was unable to detect an increase in width to compensate for the loss in length, Aumonier concluded that his sections must have increased in thickness. Conversely, altho cut celloidin sections can change in size in all directions, paraffin sections fastened relatively firmly to the slide by albumin would be free to change chiefly in thickness. Since the supporting paraffin for the section of tissue is removed before staining, reagents

should tend to shrink it more than in the case of tissue still supported by celloidin.

Generalized conclusions drawn from the specific volume changes reported in this paper should be made with caution, since these changes may be altered readily by differences in the composition and size of the tissue used, by the procedure followed, and by the reagents employed.

6. Conclusions

1. The indirect method described in the text has been found satisfactory in following volume changes of homogeneous pieces of tissue during fixation, dehydration and embedding, and area changes during sectioning, staining and mounting.

2. The changes in H-ion concentration of fixing fluids and the great variability of microscopic and volume changes produced upon rabbit kidney by the action of certain fixatives, dehydration and clearing agents, and embedding methods were determined (Tables 1, 3, 4, 5 and Fig. 2, 3).

3. Altho all reagents showed specific differences on rabbit kidney, SUSA and Bouin's fixatives yielded the best general results; and neutral formalin mixtures, especially pyridine-formalin, showed the poorest. The use of isotonic saline diluents slightly improved formalin fixation. Ten percent formalin fixation does not necessarily shrink tissues, but as shown with rabbit kidney cortex, it may cause substantial swelling.

4. Reagents producing marked decreases in tissue volume render such tissues less susceptible to further shrinkage during subsequent procedures. Shrinkage of tissue during dehydration and infiltration with hot paraffin may produce more shrinkage than fixation alone.

5. Excessive heat causes tissue distortion and shrinkage. Infiltration with hot paraffin causes considerable shrinkage, with hot nitrocellulose less, and with cold nitrocellulose the least shrinkage.

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A CONVENIENT COOLING METHOD IN PARAFFIN SECTIONING

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The cooling of the paraffin block in the preparation of microscope slides is most commonly done with a refrigerator, ice or ice water. During the past year we have utilized CO_2 for this purpose, and have found it superior on the whole to the above methods because it does away with dripping water, requires no refrigerator, and is more rapid. Refrigerators and ice have never offered a really satisfactory solution

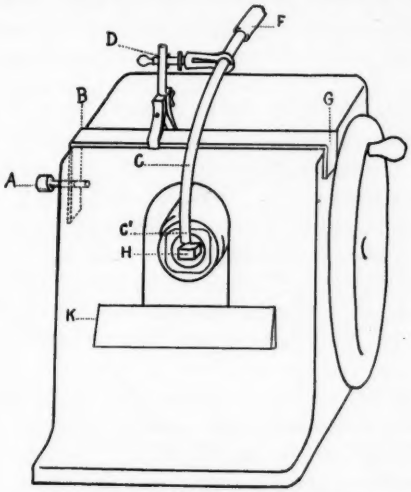

Fig. 1. Sketch of Cooling Apparatus.

(A) Screw which serves to clamp the metal strip (B, G) to the microtome. (C, C') Copper tubing, supported by a burette clamp (D), and connected to a length of rubber tubing (F). (H) Paraffin block. (K) Knife.

to the problem of cutting serial sections. Unless comparatively few sections are to be cut, the block soon becomes warm, and this means the loss of some sections, if the block is removed from the microtome for further cooling. With the device described below, the block may be cooled down as frequently as one desires without disturbing its initial orientation in the microtome. We have found the device so

easy to use that it has supplanted the refrigerator and ice for all our paraffin sections whether serial or not.

The device, shown in the accompanying figure, is exceedingly simple. A strip of metal about one-eighth inch thick and one inch wide is bent as shown at B and G so as to fit snugly over the casing of the microtome. We happen to use a Spencer (820) microtome so that only two right-angle bends are necessary. At one end of the metal strip a screw (A) permits tightening against the side of the microtome. A common burette clamp (D) is attached to the band of metal and supports a piece of one-fourth inch (outside diameter) copper tubing (C). Copper tubing of this diameter is suitable for paraffin blocks up to approximately one-fourth inch in diameter. For larger paraffin blocks the end of the copper tubing (C′) should be spread out, so that the entire block is covered by a flow of gas. A length of rubber tubing (F) leads to the needle valve on a tank of CO_2 gas. The needle valve is supplied free of charge by the company dispensing the CO_2 gas.

The paraffin block (H) is fixed as usual onto the microtome and raised to the highest position possible. The clamp at D is then adjusted so that the nozzle of the copper tube (C′) is about one-eighth inch above the paraffin block. The carbon dioxide is now turned on, allowed to flow slowly for about one-half minute, then in a strong stream for about one to two minutes. This cools the knife (K) as as well as the block itself. The gas is now shut off and sectioning may begin. If the block appears insufficiently cold at any time, it is only necessary to bring it up to its highest position and again turn on the gas.

We have used this method in cutting adult tissues and such fragile material as early amphibian eggs, using paraffin of varying melting points (45–50°C., 52–54°C., and 56–58°C.). Several persons in this laboratory have been using the same tank of CO_2 (vol. 2400 cu. in.; wt. of gas 59 lb.) for over a year and it is still three-fourths unused, so that we feel the expense for the gas is practically negligible.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

LEONARD, A. BYRON, and LEONARD, ALICE E. **A convenient turntable for staining jars.** *J. Lab. & Clin. Med.*, **26**, 562–3. 1940.

In a circular piece of wood, 1.5 in. thick and 24 in. in diameter, a depression 1 in. deep and 6 in. in diameter is cut on the lower surface to receive the pinion from the transmission gears of an old automobile. The pinion has a bearing on its lower surface which is inset into a block of wood which can be secured to a table. The turntable turns easily on its ball bearings. About 20–22 Coplin jars may be placed on the periphery. The materials cost slightly more than $1.00.—*John T. Myers.*

MILLER, ROLAND E., and MOREHEAD, ROBERT P. **An inexpensive constant temperature paraffin oven.** *J. Lab. & Clin. Med.*, **26**, 559–61. 1940.

The oven consists of a wooden box 28 in. long, 17 in. deep and 7 in. high, of 1 in. pine. It is fitted with a permanent top tapering gradually to a height of 14 in. lined with galvanized metal. One side of the box is hinged at its junction with the top. The oven has no bottom, being placed on a sheet of tin on a table. A porcelain tray 26×7 in. serves as a tray for the infiltrating work. Heat is supplied by three 100-watt bulbs set in sockets at the top of the pyramid. The thermostat is a piece of glass tubing 30 in. long and 5 mm. in diameter at one end of which is blown a ball 20 mm. in diameter. At 50 mm. from the ball the tube is bent to form a U. The tube is filled with mercury and inverted except about 10 mm. at the open end. Several drops of chloroform and a small air bubble are forced into the ball, by inversion. Mercury is removed from about 100 mm. of the open end. The tube is placed in the oven with the open end extending thru a hole in the top and the ball at the level of the paraffin. Two wires are forced thru a small cork, one wire placed below the surface of the mercury and the other a few mm. above it. These wires are attached to a Dunco relay. The temperature can be controlled by raising or lowering the wire above the mercury. The surface of the mercury should be cleaned with cotton every 2 months when in service. The oven operates on a 110 v. current and costs only about $15.00.—*John T. Myers.*

MICROTECHNIC IN GENERAL

FAVORSKY, M. V. **A new method for de-staining slides stained with iron haematoxylin.** *Compt. Rend.* (Doklady) *Acad. Sci. U.S.S.R.*, **26**, 493–4. 1940.

After removing slides from iron hematoxylin, rinsing in water and alcohol, the writer decolorizes with a 10% solution of iron alum in glycerin. This solution is placed on slide and mixed with the alcohol. Destaining is very slow, requiring several days, but the process may be watched with an oil immersion objective if necessary.—*T. E. Weier.*

FAVORSKY, M. V. **Role of clove oil in de-staining slides stained with gentian violet after Newton and its possible substitutes.** *Compt. Rend.* (Doklady) *Acad. Sci. U.S.S.R.*, **26**, 491–2. 1940.

The following substances, when diluted with alcohol to the thickness of clove oil, may be used instead of clove oil in the Newton gentian violet technic: kedrol, Canada balsam dissolved in xylol, and turpentine which has been thickened by exposure to air for several days.—*T. E. Weier.*

KRÜGER, FRIEDRICH. **Benzylbenzoat—ein geruchloses Intermedium mit günstigen Eigenschaften.** *Zool. Anzeiger*, **131**, 202–5. 1940.

Benzylbenzoate (a benzoic acid ester of benzyl alcohol) is recommended as a clearing agent after alcohol when the material is to be embedded in paraffin. Its advantages are as follows: refractive index of 1.566; boiling point higher than 150° C. (*i.e.*, in a class with cedar oil and methylbenzoate as to volatility); particularly good for whole preparations, where more volatile fluids that are incompatible with water (*e.g.*, xylene) make the tissue lose elasticity and become brittle; water-clear; colorless; neutral in reaction; not harmful to the most sensitive dyes; miscible with 90% alcohol—therefore tolerant toward water; tissues flexible after long storage; cheaper than methylbenzoate; miscible with collodion and therefore usable, instead of oil of cloves, with collodion; completely miscible with liquid paraffin but scarcely dissolving cold paraffin; not dissolving dry celloidin, but mixing freely with methylbenzoate-celloidin; valuable in clearing Spalteholz preparations. Sections impregnated by the Golgi method have been stored in it without any special precautionary methods and were unchanged after 1½ years.—*Alden B. Dawson.*

PONS, CARLOS A. **A substitute for cover glasses.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 139. 1940.

As a substitute for cover glasses the author recommends using grade 1 or heavier cellophane, cut into squares and stored in xylol. To prevent finger marks they should be removed from the xylol by forceps.—*G. H. Chapman.*

DYES AND THEIR BIOLOGICAL USES

CROSSLEY, M. L. **The sulfanilamides as chemotherapeutic agents.** *J. Amer. Chem. Soc., News Ed.*, **18**, 385. 1940.

Certain azo dyes have previously been shown to have bacteriostatic and bactericidal properties. Some of these dyes, namely prontosil and dyes of similar constitution, may be regarded as the forerunners of sulfanilamide, in that these dyes were found to break down in the animal system to give sulfanilamide, and that this product was in all cases the active element. This discovery has opened new possibilities in chemotherapy research and shifted the emphasis from dye structures to simpler molecular systems. Several hundred new sulfanilamide compounds have been synthesized and studied in experimental infections. The evidence does not permit a satisfactory correlation between the structure of the sulfanilamides and their therapeutic effectiveness. However, it is possible to say that when compared in mice with hemolytic streptococcal infection, the majority of the substituted sulfanilamides are less effective than the parent substance given in equal dosages. Any substituent on the nucleus reduces the activity. Substitution of hydrogen on the N^1 or functional group has in many cases given derivatives which are still less active, but a few are more active against hemolytic streptococcal infections and types of infections in which sulfanilamide is not particularly good: e.g. sulfapyridine (2-sulfanilamido-pyridine) has proved effective in pneumococcal infections and sulfathiazole has proved useful for the treatment of staphylococcus infections. Substitition on the N^4 nitrogen of sulfanilamide has brought about changes which result as a rule in products which are inactive. It is believed that this N^4 nitrogen is the point at which modification takes place in the animal system, transforming the drug into a harmless substance. The product formed is N^4-acetyl sulfanilamide. The amount of this product formed varies in individuals and at times in the same individual; therefore, the precise therapeutic effectiveness cannot be predicted. Much work has been done on the problem of the mode of action of sulfanilamide drugs, but all that can be said is that the major effect of the drug is to inhibit the growth of the microorganisms and this probably makes them more susceptible to destruction by the natural defense mechanism of the host. The relation between the causative agent and the biochemical changes involved in disease is a highly complex problem which must be solved if chemotherapy is ever to make headway.—*A. P. Bradshaw.*

FAVORSKY, M. V. **New polyploid-inducing chemicals.** *Compt. Rend.* (Doklady) *Acad. Sci. U.S.S.R.*, **25**, 71–4. 1939.

Seedlings of *Hordeum distichum*, var. *nutans* Schubl., were treated with the following substances: aqueous solutions of colchicine, 0.25 and 0.025%; aurantia, 0.01 and 0.001%; anilin, 0.1, 0.05, and 0.001%; trinitrophenol, 0.1 and 0.01%; and phenol, 0.1%. Tribromoanilin was dusted abundantly on the seedlings. The sparingly soluble and poorly volatile substances, diphenylamine, α and β naphthylamine and tribromophenol were applied per se. Iodoform was applied as a gas, 0.01 g. per 200 cc. of air. Nitrobenzene and dichlorodiethyl-sulphide were applied as a 1% solution in vaseline, 1 to 2 g. of the solution being used for each 200 cc. of air. The treatment lasted from 1 to 6 days. It was started either at the outset of germination or when the roots had attained a growth of 1 cm. Aurantia, diphenylamine, and tribromoanilin caused the formation of numerous polyploid cells.—*T. E. Weier.*

FRIEND, D. G. **Studies of drugs in tissue metabolism. II. Action of drugs on metabolism of tissue in serum.** *Proc. Soc. Exp. Biol. & Med.*, **45**, 140–3. 1940.

Methylene blue, at 0.5% concentration, in human serum increased the oxygen uptake of rat liver cells 50%, whereas concentrations of 0.05% and 5% increased it only 20%. The use of phosphate-Ringer's solution instead of serum, or the use of Congo red, rose Bengal-blue (information lacking as to what dye or combination of dyes this may be), brilliant vital red or Evans blue, all used clinically, failed to cause this rise. Following depression of metabolism with chloral hydrate, methylene blue increases metabolism; with sodium barbital, the dye further depresses the metabolism.—*M. S. Marshall.*

GORDON, J. **Inhibition of the anaphylactic reaction by Congo red.** *J. Path. & Bact.*, **51**, 460–3. 1940.

Guinea pigs sensitized with rabbit serum or egg albumin, then repeatedly injected with Congo red before being injected with the shocking dose of the antigen, showed fewer symptoms of anaphylactic shock than controls that had not received the Congo red. It was considered that the Congo red interfered with the entrance of antigen into the cell. The dye itself appeared toxic to a few of the animals.—*S. H. Hutner.*

NORTHEN, H. T. **Effects of methylene blue and neutral red on the structural viscosity of protoplasm.** *Trans. Amer. Micr. Soc.*, **59**, 279–80. 1940.

The effect of methylene blue and neutral red on the viscosity of protoplasm of the fresh water alga, *Spirogyra*, was measured by the displacement of chloroplasts by centrifugation at 680×gravity. Methylene blue, 0.0001% in water, did not change the viscosity of the protoplasm within 20 min. but increased it after that up to 210 min.; 0.001% methylene blue did not increase viscosity within 5 min. but did thereafter; 0.001% neutral red in water consistently increased viscosity; 0.01% neutral red decreased viscosity in the measurements made 40 and 90 min. after immersion but increased it at other intervals from 5 to 210 including the measurement at 60 min. (Source of dyes is not indicated nor whether water was distilled).—*Virgene Kavanagh.*

ANIMAL MICROTECHNIC

GINGRICH, WENDELL D. **Permanent stained preparations of thick blood films.** *J. Parasitology*, **26**, *Suppl.*, 20. 1940.

The author presents methods for the preparation of well-stained permanent thick blood films. Recommended procedure: (1) lake and stain dried blood film for 10 min. or more in dilute Giemsa stain (1–3 drops stock solution of unspecified concentration per 1 cc. distilled water), wash momentarily in distilled water and allow to dry; (2) immerse slide in May-Grunwald stain (0.5 g. in 100 cc. absolute methyl alcohol) for 30 sec.; wash momentarily in distilled water and allow to dry. Films of malarial blood, so prepared, on clean, alkali-free slides and mounted in Diaphane are as good after 2 years as at time of preparation.—*Elbert C. Cole.*

PLANT MICROTECHNIC

LEWITSKY, G. A. **A cytological study of the progeny of x-rayed Crepis capillaris Wallr.** *Cytologia*, **11**, 1–29. 1940.

Primary root tips were removed and pasted in groups on paper to be fixed and embedded as a unit.—*Virgene W. Kavanagh.*

MICROÖRGANISMS

COLE, R. M., and DAY, M. F. **The use of silver albumose (Protargol) in protozoological technique.** *J. Parisitology*, **26**, *Suppl.*, **29**. 1940.

The authors report the use of the reduced silver method of Bodian (Anat. Rec., **69**, 153, 1937) in making cover-glass preparations of protozoa. The technic demonstrates clearly the parabasal apparatus, flagella, undulating membranes and undulating cords of various protozoa. Best results were obtained with material freshly-fixed in Schaudinn's fluid or the formol-acetic No. 2 solution of Bodian.—*Elbert C. Cole.*

DELAPORTE, B. **Recherches cytologiques sur les Bactéries et les Cyanophycées.** *Rev. Gen. de Botanique*, **51**, 615–43; 689–708, 748–68. 1939. **52**, 40–8, 75–96, 112–60. 1940.

The Feulgen stain was used extensively in a cytological study of more than 50 species of bacteria and blue-green algae. Smears for Feulgen stains were usually fixed in 95% alcohol for 48 hr., hydrolyzed 10 min. at 60° C., and stained 6 hr. in the decolorized fuchsin. A positive nucleal reaction was obtained with some definite structure in each species studied altho these structures were of different shapes in the various groups. Chromosome-like bodies were found in some of the sulfur bacteria, in some oscillary parasites, and in the blue-green algae, altho the single granule or rod (axial filament) found in the other forms might be considered as a single chromosome.—*Virgene W. Kavanagh.*

ESCHE, PAUL VOR DEM. **Über auf Endoplatten violett wachsende Stuhlkeime.** *Zts. Immunitäts.*, **98**, 278–82. 1940.

Stool cultures on Endo agar sometimes yield colonies of intense purple color which is confined to the colony itself and does not diffuse thru the medium. The purple growth is lost after a few subcultures. Microscopic preparations from such purple colonies show cells with red polar granules which can be seen either unstained or when stained with methylene blue. The writer concludes that the color of the colonies is due to the storage of fuchsin in the organism in characteristic polar bodies.—*H. J. Conn.*

KONSCHEGG, TH. **Ersparnisse bei der Gram-Färbung.** *Münch. Med. Woch.*, **87**, 456–7. 1940.

A saturated aqueous picric acid solution is recommended as a mordant in place of the usual iodine solution in the Gram-staining procedure. The saturated solution is used directly when freshly prepared. After a few weeks, this solution is diluted with an equal volume of distilled water before use.

The author also uses a primary staining solution which is prepared as follows: Shake well 3 ml. of anilin oil, 7 ml. of 95% ethanol and 90 ml. of distilled water and filter the mixture thru a wet filter paper. To the filtrate add 2 g. of gentian violet, allow the mixture to stand 24 hr. and then filter.

The staining is carried out as follows: Stain with the above solution, 5 sec.; treat with either saturated or diluted picric acid solution, 5 sec.; decolorize with alcohol; wash with distilled water; counterstain with carbol fuchsin or any other satisfactory stain.—*L. Farber.*

LEVINE, NORMAN D. **Changes in the dimensions of Balantidium from swine upon cultivation.** *Amer. J. Hyg.*, **32**, *Section C*, 1–7. 1940.

The following technic is employed: Examine cecal material from swine just killed, for *Balantidium.* If present, place a small amount of the material in a relatively large amount of Klemmenberg's fixative (saturated picric acid in 2% H_2SO_4). Allow the fixed protozoa to settle. With a pipette, place drops on

slides, apply cover slips and seal with vaseline. Measure 100 individuals with a calibrated 5× ocular and a 4 mm. apochromatic objective. At the same time inoculate 10 tubes of Glaser and Coria's medium (3% horse serum in Ringer's solution with a little powdered rice starch). After cultivation measure the organisms in the same way. The mean length-width ratio becomes smaller, hence *Balantidium suis* may come to resemble *Balantidium coli.*—*John T. Myers.*

MAYFIELD, CATHERINE R., and GOBER, MAUD. **Comparative efficiency of Endo, lithium chloride Endo, desoxycholate-citrate, and bismuth sulfite media for the isolation of Eberthella typhosa.** *Amer. J. Pub. Health*, **30**, 69–76. 1940.

These media were compared for the isolation of typhoid bacteria from feces, typical colonies then being put thru agglutination and fermentation tests. The specimens were inoculated on the media when received, then placed at 20° C. and inoculated again on the second and third days. Plates were incubated for 18 hr. at 37° C. The desoxycholate agar was a dehydrated product of the Baltimore Biological Laboratories; the plain Endo, LiCl Endo, and bismuth sulfite agars were Difco products. Plain Endo and LiCl Endo as prepared by the Mississippi State Board of Health Laboratories were also used. The medium of the latter laboratory consisted of peptone, 10 g., beef extract, 5 g., agar, 30 g., in 1000 cc. water, and adjusted to pH 7.8–8.0. To this was added 1% lactose, and 1 cc. of a 10% Na_2SO_3 solution to which 0.5 cc. of a saturated alcoholic solution of basic fuchsin had been added. The Difco Endo had a slightly different composition. LiCl was added in 0.5% concentration to the plain Endo media. For isolation, LiCl Endo was superior to plain Endo. Comparing LiCl Endo, desoxycholate-citrate and bismuth sulfite, 74% of all the positives (724) were found on the first, 82% on the second, and 83% on the third. Of these 724 positives, 195 were positive only on one medium; 42 on LiCl, 65 on desoxycholate-citrate, and 88 on bismuth sulfite. There were 127 specimens on LiCl, 144 on desoxycholate-citrate, and 246 on bismuth sulfite which were positive on the second and third day's plating that would have been reported negative if only one day's plating had been made. As between specimens examined for diagnosis, release and carriers, there was no marked difference in the efficiency of the three media.—*M. W. Jennison.*

MOORE, MORRIS. **The chorio-allantoic membrane of the developing chick as a medium for the cultivation and histopathologic study of pathogenic fungi.** *Amer. J. Path.*, **17**, 103–20. 1941.

Fertile hen's eggs were incubated 10–14 days at approximately 33° C. and inoculations made directly into the chorio-allantoic membrane. Successful growth was obtained with 15 pathogenic fungi. Protocols are given for the type of growth and lesions produced by each organism, and the results illustrated by 29 figures.—*H. A. Davenport.*

MORISON, J. E. **Capsulation of haemolytic streptococci in relation to colony formation.** *J. Path. & Bact.*, **51**, 401–12. 1940.

Exceptionally clear preparations of capsule formation by streptococci can be obtained from colonies grown on sterile No. 300 cellophane (a very thin grade). The cellophane is sterilized by autoclaving between sheets of blotting paper in a separate petri dish, and then dropped aseptically on blood agar. The inoculum is placed upon the cellophane. Capsule staining was most successful when the method of Butt *et al* (J. Inf. Dis., **58**, 5, 1936) was followed: Mix a loopful of a dense suspension of organisms with a loopful of 6% glucose. Then mix in a loopful of India ink, and spread like a blood film. Dry without heat. Stain with alkaline methylene blue.

Intact colonies can be stained on the cellophane with most ordinary dyes without objectionable staining of the cellophane.—*S. H. Hutner.*

RYU, EIHYO. **Studies on the simplification of special staining methods of bacteria. I.** *Kitasato Arch. Exp. Med.*, **17**, 53–7. 1940.

The following simple method for staining the metachromatic granules of the diphtheria bacillus is described: Fix culture with flame or methyl alcohol. Stain

2 or 3 min. without heating in a solution of 5 cc. saturated aqueous $K_2Al_2O_4$ and 1 cc. of saturated alcoholic crystal or methyl violet. Wash well in tap water. The metachromatic granules stain dark purple and the bacterial bodies light purple.

A simple method for staining granules of bacteria is described as follows: Place a loopful of phenol on the slide for a mordant, and smear the culture in it. (For Gram-negative organisms 4–5% aqueous phenol is used, and 7% aqueous phenol for Gram-positive organisms). Dry by flame and fix. Stain 10–30 sec. at room temperature in Ziehl's carbol fuchsin diluted 10 times (Pfeiffer's solution). Wash with water. If a counterstain is desired, stain 30 sec. with Loeffler's methylene blue and wash with water before staining with carbol fuchsin.

Tubercle bacilli may be stained as follows: Place one or two loops of KOH on a slide; mix sputum or culture, and smear; dry and fix. Place a few drops of 0.05% fuchsin on slide, and pass over flame until it boils. After 20–40 sec., wash lightly with water. Stain 30 sec. at room temperature with 2% methylene blue. Wash with water. Examination of sputum of 80 tubercular patients by this method showed 69 positives and 11 negatives. Ziehl-Neelsen's method showed 58 positives and 22 negatives. More organisms per field were demonstrated by the former method than with Ziehl-Neelsen's method.—(*From Biological Abstracts*).

RYU, EIHYO. **On a simplified method of staining acid-fast bacteria. II.** *Jap. J. Vet. Sci.*, **2**, 488–90. 1940.

Smears of sputum or culture are dried and fixed by flaming, then flooded with carbol fuchsin and heated for 30–60 sec. After washing with water, they are counter-stained with 2% aqueous malachite green for 1 min. No decolorization is used.—*A. G. Karlson* (*from Biological Abstracts*).

RYU, EIHYO. **On the Gram-differentiation of bacteria by the simplest method. III. The sulphuric acid method.** *Jap. J. Vet. Sci.*, **2**, 491–6. 1940.

A half loopful of young agar-slant culture is mixed with half a drop of concentrated H_2SO_4 on a glass slide and mixed thoroly with a platinum loop. The writer claims that Gram-negative bacteria dissolve instantly and the suspension becomes transparent. Gram-positive forms show no change and there remains a turbid suspension.—*A. G. Karlson* (*from Biological Abstracts*).

SALLE, A. J., SHECHMEISTER, I. L., and McOMIE, W. A. **Germicidal efficiency of some medicinal dyes compared to a group of non-dye disinfectants.** *Proc. Soc. Exp. Biol. & Med.*, **45**, 614–7. 1940.

Fifteen dyes have been tested by the method of comparing toxicity for chick heart tissue and purported bactericidal action. Only the triphenyl methane dyes were found to kill both the typhoid bacillus and staphylococci and of these only brilliant green killed *E. typhosa* under the conditions tested.—*M. S. Marshall.*

SCHALLOCK, G. **Vereinfachter Nachweis von Tuberkelbazillen in histologischen Schnitten durch die Fluoreszenmethode nach Hagemann.** *Münch. Med. Woch.*, **87**, 102–3. 1940.

Paraffin sections for demonstration of the tubercle organism are treated as follows: Remove paraffin and treat with absolute alcohol, 95% alcohol, 75% alcohol and distilled water. Stain with 1:1000 auramin, for 15 min. at 37° C.; decolorize with alcoholic HCl (concentration not stated) for 1–3 min. at 37° C.; wash in distilled water; immerse in thiazol yellow 1:1000, 2 sec.; wash in 50% alcohol and in distilled water. Frozen sections are freed from fat before auramin treatment with 95% alcohol or chloroform for a few minutes followed by washing with distilled water.

The tubercle bacilli can be distinguished from the weakly bluish fluorescent background. This method is satisfactory only for material which has been fixed not more than 4–5 days in formalin.

Sections stained with hematoxylin-eosin can be decolorized by immersion for 5–10 min. in a mixture of alcoholic HCl (concentration not given) and 3–5 ml. of 25% H_2SO_4 followed by a wash in distilled water. The decolorized sections can then be stained with auramin etc.—*L. Farber.*